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Large Scale Partial Purification and Molecular and Kinetic Properties of the (Na + K)-activated Adenosine Triphosphatase from *Artemia salina* Nauplii*

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The sodium-potassium coupled transport enzyme (NaK-ATPase) has been partially purified from the nauplius larva of the brine shrimp, *Artemia salina*, by treatment of isolated membranes with deoxycholate, followed by a new freeze-thaw procedure. The enzyme remains in the particulate or vesicular state throughout and is stable. Purification is up to 200-fold (relative to the homogenate), with a yield of 10 mg of protein from 200 g, wet weight, of brine shrimp nauplii. The enzyme preparations have specific activities up to 475 μmol of P_i/h/mg of protein, with an estimated 50% purity, assuming both the large and small subunits are the protein constituents of the holoenzyme. The kinetic properties of the brine shrimp NaK-ATPase are not altered during purification. Two proteins enrich during purification, the larger of which is identified as the catalytic subunit by its potassium-sensitive, (Na + Mg)-dependent phosphorylation from [γ-^{32}P]ATP. The smaller subunit stains positively with the periodic acid-Schiff reagent, indicating it is a glycoprotein. On sodium dodecyl sulfate-polyacrylamide gels, the larger has an apparent molecular weight of 95,000 to 101,000 and the smaller an apparent molecular weight of 38,000 to 40,000. The mass ratio is estimated to be greater than two, corresponding to a molar ratio of one large to one small subunit. Binding studies indicate that the preparation has an equivalent number of phosphorylation sites and ATP binding sites. The corresponding turnover numbers from the binding studies are 3210 min^{-1} and 3048 min^{-1}. The calculated molecular weight of a theoretically purified enzyme based on the binding studies is 230,000 and 220,000, respectively, for the two ligands. The binding studies and the subunit molecular weight and mass ratio analysis suggest that the enzyme is an $\alpha_2\beta_2$ tetramer, of which only half of the sites are apparently reactive at one time. The subunits are consistently double-banded on sodium dodecyl sulfate gels, and the potassium-sensitive phosphorylation reaction appears in both bands of the large subunit. The relative amount of each band in the doublet of the large subunit is correlated with two membrane populations of different sedimentation properties and two unique total monovalent cation activation curves (increasing ionic concentration at constant Na/K ratio). The evidence suggests there may be two NaK-ATPases in the brine shrimp.

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The sodium-potassium transport system, sodium-potassium-activated adenosine triphosphatase (NaK-ATPase)[1] is an intrinsic membrane protein that requires lipid in order to function (1–3). Purification of the NaK-ATPase has been achieved from mammalian kidney (4–6), dogfish rectal gland (7), electric eel electroplax (8), and duck salt gland (9). These studies indicate that the NaK-ATPase consists of two different protein subunits. On SDS-polyacrylamide gels, the larger subunit has a molecular weight of 90,000 to 100,000, and the smaller, a glycoprotein, 47,000 to 60,000.

Our interest is to study the biogenesis of the NaK-ATPase. Many of the tissues from which the NaK-ATPase has been purified are not readily adaptable to the necessary biochemical manipulations in a laboratory situation and vice versa. Larval brine shrimp, *Artemia salina*, are quite suitable for laboratory experimentation, and we have found that the nauplius contains high levels of NaK-ATPase activity (10). More importantly, we have found that activity develops from undetectable levels 24 h earlier in development (11). To further establish the larval brine shrimp as a model system for the study of the biogenesis of the NaK-ATPase, it remains necessary to purify the enzyme from this source.

This paper describes techniques for large scale isolation of brine shrimp naupliar membranes that are rich in the NaK-ATPase and the subsequent partial purification (up to 200-fold over the homogenate) by deoxycholate and a freeze-thaw procedure. The partially purified preparation is stable and in high yield (about 10 mg of protein/200 g of starting material) at a purity of 30 to 50%. The molecular and kinetic properties of this preparation are examined and compared to other preparations derived from vertebrate sources. A preliminary report of this work has been published (12).

MATERIALS AND METHODS

Source and Rearing of Brine Shrimp Nauplii—Dried brine shrimp "eggs" (gastrulae), from Great Salt Lake, Utah, were purchased from Longlife Products, Harrison, N. J., and stored at −20°C. Three

[1] The abbreviations used are: NaK-ATPase, (Na + K)-activated adenosine triphosphate phosphohydrolase (EC 3.6.1.3); SDS, sodium dodecyl sulfate; HM, homogenizing medium; FT-membranes, freeze-thaw-treated membranes.

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hundred grams of dry cysts were hydrated in tap water at 5°C for 3 to 4 h and those which sank were washed several times in tap water. The washed, hydrated cysts were divided among 12 2800-ml Fernbach flasks containing 500 ml of artificial sea water (Instant Ocean Sea Salts), and then incubated at 26°C with continuous shaking for 40 to 48 h. Hatched nauplii were harvested from the remaining shells and unhatched cysts by the method of Finamore and Clegg (13). This produced a harvest of 170 to 210 g, wet weight, of nauplii.

Large Scale Preparation of NaK-ATPase-rich Membranes—Brine shrimp nauplii were homogenized with two strokes of a 55-ml Vitro glass-Teflon homogenizer at about 2500 rpm at a ratio of 1 g of nauplii to 2.5 ml of homogenizing medium (HM). HM consisted of 250 mM sucrose, 100 mM imidazole/HCl buffer, pH 7.2, 2 mM Na_2EDTA, and 2 mM 2-mercaptoethanol. The homogenate was fractionated by differential centrifugation in a Sorvall RC2-B refrigerated centrifuge. The homogenate was centrifuged at 500 × *g* for 5 min in the Sorvall GSA rotor, and the supernatant was decanted and saved. The pellet was resuspended in one-half of the original volume of HM with two strokes of the Vitro homogenizer and centrifuged at 500 × *g* for 5 min. The resulting supernatant was combined with the previous one and centrifuged at 1,000 × *g* for 15 min in the GSA rotor. The pellet was washed as before in one-fourth of the original volume of HM and recentrifuged at 1,000 × *g* for 15 min. The combined supernatants from these two centrifugations were then centrifuged at 27,000 × *g* for 120 min in the GSA rotor. Following centrifugation, the supernatant was decanted carefully, leaving the last several milliliters with the pellet to avoid loss of the lighter buff colored sediment. This pellet contains the desired membranes as well as contaminating mitochondrial membranes that tend to layer in the lower portion of the pellet (10) as a darker colored sediment. Additional HM was added to the high speed pellets and the upper lighter colored membrane layer was removed by gently swirling it off or by dissecting it off with a glass rod. Complete separation of the heavier mitochondrial layer from the lighter membranes required two and sometimes three centrifugations at 48,000 × *g* for 30 min in a Sorvall SS-34 rotor. Resuspension of the upper layer at each stage was done with a single stroke of the Vitro homogenizer in decreasing total volumes of the suspension, beginning with about 200 ml. The final membrane pellet was resuspended in HM to give a total volume of about 25 ml (10 to 12 mg of protein/ml).

Partial Purification of NaK-ATPase from Isolated Membranes—The first step in the purification of the NaK-ATPase from the brine shrimp membrane preparation involved treatment with the detergent deoxycholate. The membranes were diluted 1:1 (v/v) with 0.70% deoxycholate, allowed to stand at room temperature for 30 min, then centrifuged at 48,000 × *g* for 2 h in a Sorvall SS-34 rotor. The supernatant was carefully aspirated off to avoid loss of the flocculent pellet and discarded. The pellet was resuspended in HM to a total volume of 12 to 15 ml (3 to 5 mg of protein/ml) and then frozen at −12°C. This preparation is referred to as the deoxycholate membranes and can be stored frozen up to 1 month without loss of enzyme activity.

The frozen deoxycholate membranes were thawed slowly at room temperature and only very gently mixed. They were then layered on a discontinuous sucrose gradient prepared as follows: 5 ml of 28% (w/v) sucrose, 7 ml of 18% (w/v) sucrose, sample (up to 20 mg of protein), and distilled water overlay to give a final volume of 25 ml per Beckman 25.1 cellulose acetate gradient tube. The sucrose in each case also contained 1 mM Na_2EDTA and 30 mM imidazole/HCl buffer, pH 7.2. One enzyme preparation was usually loaded in three gradient tubes. The gradients were centrifuged at 22,000 rpm for 25 min, using a Beckman SW 25.1 rotor. The final freeze-thaw enzyme preparation (FT-membranes) bands at the interface between the 18 and 28% sucrose layer and was collected by aspiration. The material penetrating the 28% sucrose layer and forming a light layer over the top of the pellet was recovered by decanting and was included in the final FT-membrane preparation.

SDS-Polyacrylamide Disc Gel Electrophoresis—The procedure of SDS gel electrophoresis was taken from the Burgess modification of the Laemmli (14) method and used as described by Hokin *et al.* (7). The protein bands were stained with Coomassie brilliant blue. Glycoproteins were detected by periodic acid-Schiff (PAS) staining techniques modified from Zacharius *et al.* (15) and Fairbanks *et al.* (16). Fading required that the PAS gels be photographed within 1 week after preparation.

Molecular weight calibration of the SDS-polyacrylamide gels was done with the following markers: β-galactosidase subunit, 130,000; phosphorylase A, 92,500; bovine serum albumin, 68,000; catalase, 57,500; pyruvate kinase, 57,200; glyceraldehyde-3-phosphate dehydrogenase, 37,000; hexokinase, 27,500; ribonuclease A, 13,700; cytochrome *c*, 11,700; and insulin, 5,700.

Gel scanning was performed with a photovolt Densicord densitometer on Response 5, which approximates an absorption scale. For densitometric quantification, four protein standards of different molecular weights were examined (phosphorylase A, bovine serum albumin, glyceraldehyde-3-phosphase dehydrogenase, and ribonuclease A), in the manner of Fishbein (17). Linearity was found for the standards between 1 and 30 μg of protein.

Phosphorylation with [γ-^{32}P]ATP—The optimum conditions for phosphorylation of the brine shrimp NaK-ATPase were characterized for $MgCl_2$, NaCl, and KCl concentrations and for time of incubation. The optimum phosphorylation reaction was run at 0°C with 2.0 μM [γ-^{32}P]ATP (10^{14} cpm/mol), 0.2 mM $MgCl_2$, 100 mM NaCl, 100 mM imidazole/HCl buffer (pH 7.2), and 50 μg/ml of enzyme protein. Nonspecific phosphorylation was estimated by an identical reaction mixture except that 20 mM KCl was substituted for the 100 mM NaCl. The reactions were initiated by addition of an enzyme buffer solution and terminated at 20 s by addition of one reaction volume of 5% trichloroacetic acid solution containing 0.6 mM Na_2ATP and 0.6 mM NaH_2PO_4. The isolation of the phosphorylated intermediate of the NaK-ATPase was done by either a centrifugation method or a filtration method. For the centrifugation method, a total reaction volume of 1 ml was used. After addition of 1 ml of the trichloroacetic acid solution, the protein precipitate was allowed to form for 10 min at 0°C, then centrifuged at 48,000 × *g* for 5 min. The pellet was washed with 2 ml of the trichloroacetic acid solution and centrifuged at 48,000 × *g* for 5 min. The pellet was then dissolved in 0.5 ml of 0.5 M NaOH for assay of radioactivity or protein (18). Protein recovery was 81.6 ± 1.9% ($\bar{X}$ ± S.E., n = 6). For the filtration method, a total reaction volume of 0.25 ml was used. The reactions were terminated with 0.2 ml of the trichloroacetic acid solution and 0.05 ml of 5 mg/ml of bovine serum albumin was added as carrier protein. After 10 min, the precipitate was filtered through 0.45 μ Millipore filters (25 mm diameter) and washed with three 2-ml aliquots of the trichloroacetic acid solution. Protein recovery was assumed to be 100%. The sample filter discs or sample solutions were counted with 15 ml of a BBOT toluene (16 g of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene/liter of toluene):Methyl Cellosolve (5:3) scintillation fluid in a model 3310 Packard Tri-Carb liquid scintillation spectrometer.

For processing on SDS-polyacrylamide gels, the phosphorylation reaction mixture consisted of 100 μM [γ-^{32}P]ATP, 1 mM $MgCl_2$, 100 mM NaCl (or 20 mM KCl), 100 mM imidazole/HCl buffer (pH 7.2), and FT-membrane enzyme preparation at a protein concentration of 1 mg/ml. The reactions were run at 0°C for 20 s and stopped with an equal volume of ice cold double strength concentrated electrophoresis sample buffer (7). The solubilized protein was applied to SDS-polyacrylamide gels (about 50 μg/gel) and processed as above except that the chamber temperature was maintained between 0–4°C. The gels were run in duplicate, one set stained for protein, the other sliced (0.5 mm thickness) and counted by liquid scintillation.

[^{14}C]ATP Binding—Binding of [^{14}C]ATP to the NaK-ATPase preparations was determined similarly to the procedure described by Jørgensen (19). The sodium salt of [^{14}C]ATP (407 mCi/mmol, New England Nuclear) was purified and converted to the Tris salt according to Nørby and Jensen (20). Reactions were run in a total volume of 1 ml containing 0.24 to 0.66 mg of protein, 30 mM Tris/EDTA (pH 7.5) 80,000 cpm of [^{14}C]ATP, and unlabeled Tris/ATP (Sigma). The ATP concentration was varied in six reaction centrifuge tubes at 0.1, 0.2, 0.4, 0.6, 0.9, and 1.2 μM. Two other tubes contained 3 mM ATP to control for unbound ATP. The tubes were centrifuged at 100,000 × *g* for 30 min directly after addition of the enzyme preparation. The supernatant was removed with a Pasteur pipette and 0.05-ml aliquots were counted by liquid scintillation. The pellet was dissolved in 0.5 ml of 0.5 M NaOH at 37°C for 30 min and 0.1-ml aliquots were assayed for radioactivity by liquid scintillation and for protein by the method of Lowry *et al.* (18). Protein recovery in the pellets was 104.1 ± 4.0% ($\bar{X}$ ± S.E., n = 8). The binding parameters were estimated from Scatchard plots by fitting linear regression lines by the method of least squares.

Comparison of Kinetic Properties of Membrane and Partially Purified NaK-ATPase—Temperature, pH, substrate, and monovalent cation stimulation characteristics were examined similarly to previous kinetic characterization studies (10). In the case of the temperature profile studies, a deoxycholate membrane preparation was examined in addition to the membrane and FT-membrane preparations. The pH profile was examined in a 25 mM histidine, 25 mM

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imidazole buffer system. The substrate curves were obtained using a constant Mg:ATP ratio of 2:1, and the reactions were run for 10 min. The monovalent cation activation characteristics investigated were variable ionic strengths at a constant Na:K ratio of 4:1 and variable Na/K ratios at a constant ionic strength of 160 mM (total Na + K). Inorganic phosphate was measured either by the method of Ernster *et al.* (21), or by the method of Huxtable and Bressler (22). Protein was estimated by the method of Lowry *et al.* (18).

RESULTS

Isolation of NaK-ATPase-rich Membranes—Fractionation of the brine shrimp naupliar homogenate by differential centrifugation results in the distribution of NaK-ATPase and protein in the various fractions as shown in Table I for a typical preparation. Activity of the enzyme in the homogenate was difficult to assess due to its crude nature, resulting in an underestimate of its total activity. Approximately one-third of the total enzymatic activity in the embryo was recovered in the final membrane fraction. From six experiments, the average total yield of NaK-ATPase in the membrane fraction was 69.1 ± 6.0 μmol of P_i/h/g of nauplii ($\bar{X}$ ± S.E.). These membranes had about a 20-fold increase in the specific activity of NaK-ATPase over that in the homogenate, averaging 39.0 ± 2.4 μmol of P_i/h/mg of protein ($\bar{X}$ ± S.E., $n = 6$).

Partial Purification of NaK-ATPase from Isolated Membranes—An example of the partial purification of the brine shrimp NaK-ATPase by the deoxycholate and freeze-thaw treatments is shown in Table II. Approximately one-third of the enzyme in the membrane preparation is recovered in the final FT-membranes, with specific activities routinely better than 250 μmol of P_i/h/mg of protein. The preparation shown in Table II is particularly good due to a high specific activity increase in this case at the deoxycholate step. The relative increase in specific activity at the deoxycholate step normally ranged from 3.5- to 4.0-fold, of which about 2.5- to 3.0-fold is due to removal of non-enzyme protein from the membranes, the remainder being due to detergent activation. A peculiarity of the brine shrimp system is that deoxycholate activation of the enzyme is only seen after the deoxycholate-treated membranes have been centrifuged free of deoxycholate and resuspended in deoxycholate-free media. Activation amounts to a maximum of 160%, while the activity of the enzyme in the presence of deoxycholate is about 90% of its pretreatment value. The freeze-thaw step results in a 2 to 3-fold increase in specific activity, all of which is due to purification and not activation. The higher specific activity preparations are always obtained from the material at the 18 to 28% sucrose interface of the discontinuous gradient. The enzyme remains in the vesicular or particulate state throughout the purification procedures. The deoxycholate treatment solubilizes a substantial amount of the contaminating proteins, and the freeze-thaw procedure appears to produce a population of small vesicles that are particularly rich in the NaK-ATPase and can be isolated free of the other membrane fragments.

TABLE I

Distribution of protein and NaK-ATPase from 200 g, wet weight, of brine shrimp nauplii fractionated by differential centrifugation

Fraction	Total		Specific activity	Enzyme yield
	Protein	NaK-ATPase		
	mg	*μmol P_i/h*	*μmol P_i/h/mg*	%
Homogenate	14,600	28,200	1.94	100.0
500 × *g* pellet	7,760	15,800	2.04	56.0
1000 × *g* pellet	380	1,580	4.14	5.6
High speed				
Lower pellet	620	5,540	8.81	19.6
Membranes	340	13,500	40.0	47.8
Supernatant	3,280	6,180	1.88	21.8

TABLE II

Purification of NaK-ATPase from membranes prepared from 210 g, wet weight, of brine shrimp nauplii

Purification step	Total		Specific activity	Enzyme yield
	Protein	NaK-ATPase		
	mg	*μmol P_i/h*	*μmol P_i/h/mg*	%
Membranes	370.00	12,700	34.3	100.0
Deoxycholate membranes	47.40	8,510	179.0	67.0
FT-membranes				
18 to 28% Interface	2.59	1,330	514.0	10.5
Upper pellet	8.44	2,410	286.0	19.0

The final partially purified preparation represents a relative purification of 100- to 250-fold over the homogenate.

Stability of Brine Shrimp NaK-ATPase—As noted above, the deoxycholate membrane preparation can be stored frozen in HM at −12°C for 3 to 4 weeks without loss of enzyme activity (defined as 95 to 100% of original activity). The membrane preparation can be similarly stored. The membrane, deoxycholate membrane, and FT-membrane preparations retain their activity for up to 1 week at 0°C. If the FT-membrane enzyme preparation is isolated from deoxycholate membranes which have been frozen for 3 weeks, their stability at 0°C may be limited to 3 or 4 days. Loss of activity after this period is 5 to 10%/day.

Electron Microscopy of Partially Purified Brine Shrimp NaK-ATPase—Electron photomicrographs of two FT-membrane enzyme preparations are shown in Fig. 1. The FT-membranes from the discontinuous sucrose gradient were diluted 1:1 with 30 mM imidazole/HCl buffer (pH 7.2) and centrifuged at 48,000 × *g* for 1 h. The upper portion of the resulting pellet is shown in Fig. 1*A* and the lower portion in Fig. 1*B*. The membranes in Fig. 1*A* appear relatively uniform and frequently show one membrane ring within another. The membrane rings appear complete, suggesting that this preparation consists of closed vesicles which may be collapsed within themselves or frequently as one vesicle within another. The membranes in Fig. 1*B* are more irregular, with inclusion of complex membrane-within-membrane arrays and the appearance of more numerous small vesicular structures. These complex structures may contain some latent activity if some NaK-ATPase units are trapped within them. The enzyme at this stage, however, is unstable to a second even mild detergent treatment.

SDS Gel Electrophoresis of Brine Shrimp NaK-ATPase at Different Levels of Purification—Fig. 2 demonstrates the protein patterns of the deoxycholate membrane (*A*) and FT-membrane (*B* to *F*) enzyme preparations on SDS-polyacrylamide disc gels. Gel patterns *B* and *C* represent FT-membrane preparations that correspond to those shown in the electron micrographs of Fig. 1, *A* and *B* (but were prepared at different times). *Gels B* and *C* are from the lower and upper layers, respectively, of the pellet obtained from sedimenting the discontinuous sucrose gradient FT-membranes. Two protein regions, designated *a* and *b* in Fig. 2, are observed to enrich with increasing specific activity of the enzyme. *Regions a* and *b* both contain two protein bands, although the double banding is not as clear in *Region b* due to greater diffusion of lower molecular weight proteins. The molecular weights for these bands are, in descending order: 99,000 to 103,000, 93,000 to 97,500, 39,000 to 41,000, and 37,000 to 38,500. The doublet banding is always observed, while other proteins in the same gel do not appear to follow this behavior. No additional bands are resolved among the doublets of *Region a* after prolonged

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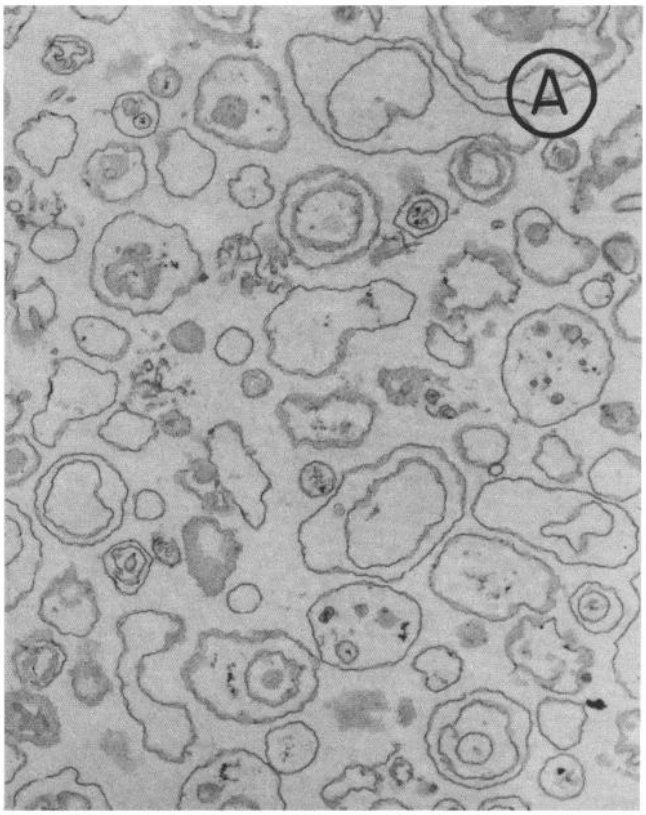

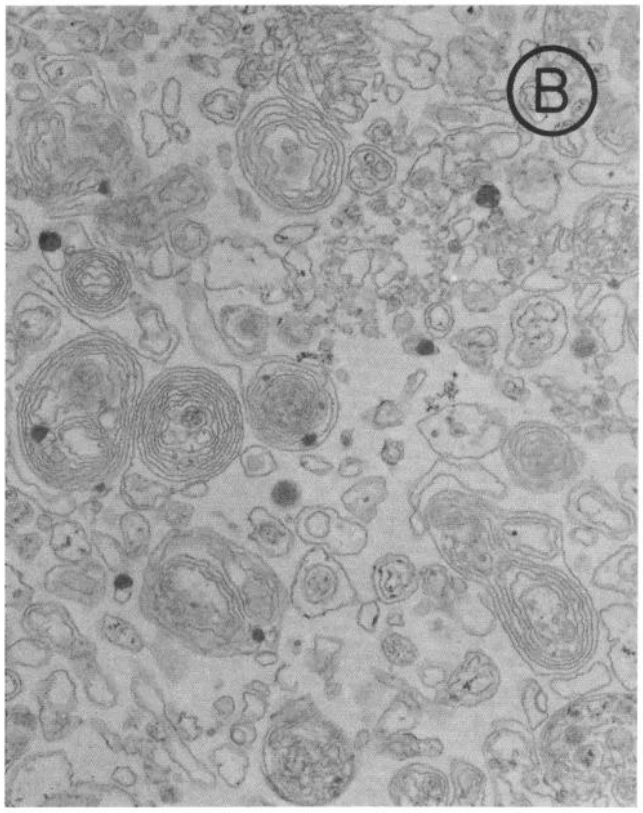

FIG. 1. Electron micrographs of the FT-membrane enzyme preparation. The preparation was sedimented by centrifugation at 48,000 × *g* for 1 h which produced a layered pellet. The upper layer (*A*) (× 16,000) had a specific activity of 356 μmol of P_i/h/mg of protein and the lower layer (*B*) (× 15,800) had a specific activity of 273 μmol of P_i/h/mg of protein. The pellets were fixed in 5% glutaraldehyde containing 250 mM sucrose, 150 mM cacodylate buffer (pH 7.5), and postfixed with 1% O_sO_4 containing 100 mM phosphate buffer (pH 7.2). The specimens were embedded in Araldite, thin sectioned, and stained with uranyl magnesium acetate and lead citrate for examination with an RCA EMU 3H electron microscope.

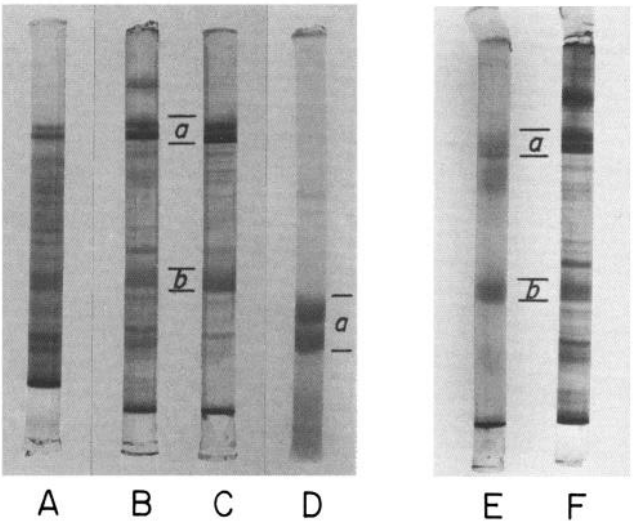

FIG. 2. SDS-polyacrylamide gels illustrating the protein composition of the deoxycholate membrane (*A*) and FT-membrane (*B* to *F*) enzyme preparations. *Gels B* and *C* represent the lower and upper layers of the pellet (specific activities, 222 and 475 μmol of P_i/h/mg of protein, respectively) obtained after sedimenting the discontinuous sucrose density gradient FT-membranes (48,000 × *g*, 1 h). Gel pattern *D* was from prolonged electrophoresis (11 h) in which the protein bands of *Region a* had migrated to the bottom of the gel. *Gels A* to *D* and *F* were stained for total protein with Coomassie brilliant blue. *Gel E* is identical with *Gel F* except that it was stained for glycoproteins by the PAS reaction.

electrophoresis (see *Gel D*, Fig. 2). Changes in the pH (6.8 and 7.8) of the running gels, in the buffer (borate), or in the dispersing agent (SDS plus urea) do not alter the double banding behavior.

Identification of NaK-ATPase Subunits on SDS Gels—Fig. 2*E* shows a PAS-stained SDS gel from an FT-membrane enzyme preparation with a specific activity of 222 μmol of P_i/h/mg of protein in comparison to a Coomassie brilliant blue-stained gel (Fig. 2*F*) from the same enzyme preparation. Both *Regions a* and *b* show a positive reaction to PAS while most other proteins in the gel are negative. The intense PAS-stained band at the tracking dye may be small glycoproteins or glycolipids (7). *Region b* is presumably the small glycoprotein subunit of the NaK-ATPase. The positive PAS reaction in *Region a* is seen in all preparations examined, even with specific activities of 475 μmol of P_i/h/mg of protein. The positive PAS reaction in *Region a* is not due to high protein density, since the intensely stained band above *Region a* is PAS-negative. This suggests that the brine shrimp NaK-ATPase large subunit contains carbohydrates. The relative PAS staining intensities of *Regions a* and *b* could not be determined as the reaction in *Region a* appeared to develop faster and fade faster than that in *Region b*.

Identification of the phosphorylated subunit of the brine shrimp NaK-ATPase is shown in Fig. 3. A sodium-dependent potassium-sensitive peak in ^{32}P radioactivity is observed to occur over both bands of the *Region a* protein. In some other preparations, the profile appears as a single peak with a shoulder instead of as two separate peaks, as a result of the difficulty in obtaining a section between the two bands. Attempts to increase the separation of the doublets by prolonged electrophoresis resulted in dephosphorylation of this labile intermediate. Although it is clear that both bands of *Region a* are phosphorylated and thus identifiable as the catalytic

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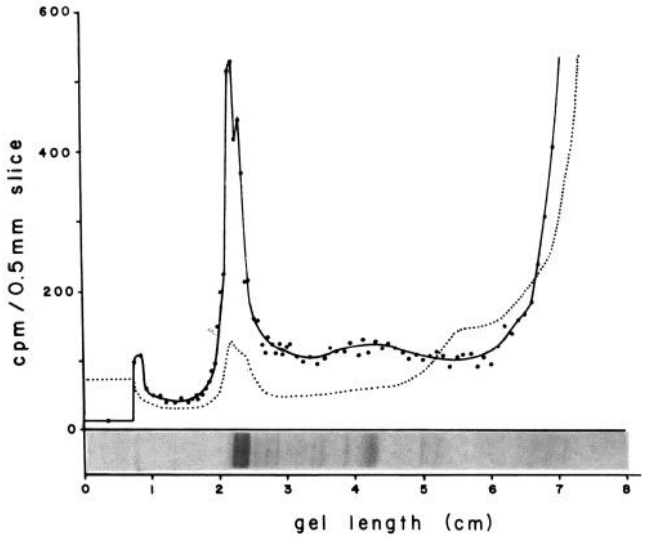

FIG. 3. Distribution of radioactivity in SDS-polyacrylamide gels prepared from FT-membrane enzyme phosphorylated with [γ-^{32}P]-ATP in the presence of sodium (*solid line*) or potassium (*broken line*). A Coomassie brilliant blue-stained sodium gel is shown below.

subunit(s), it is not possible because of potential technical artifacts to make an evaluation of the relative phosphorylation of the two bands. The radioactivity at the bottom of the gels depicted in Fig. 3 is due to unreacted [γ-^{32}P]ATP and free $^{32}P_i$.

Thirty acrylamide gel patterns from FT-membrane preparations with specific activities between 200 and 475 μmol of P_i/h/mg of protein were examined for the quantitative characteristics of the catalytic and glycoprotein subunits. The mass ratio of the two subunits (ratio of *Region a* to *Region b*) was 1.99 ± 0.22 ($\bar{X}$ ± S.E.). Estimates of the specific activity of a pure enzyme preparation based on the amount of protein contamination (all protein except that in *Regions a* and *b*) was 833 ± 45 μmol of P_i/h/mg of protein ($\bar{X}$ ± S.E.). The amounts of protein in the slower and faster bands of a doublet in either *Region a* or *b* were variable from preparation to preparation, but did not correlate with mass ratio (large subunit/small subunit), measured specific activity, or estimates of pure enzyme specific activity. The ratio of the slower band to the faster band in *Region a* to the same ratio in *Region b*, however, gave a significant ($p \ll 0.001$) positive correlation.

Binding Studies—The number of binding sites for phosphate was estimated from studies of phosphorylation of the NaK-ATPase by [γ-^{32}P]ATP using techniques of Millipore filtration and centrifugation. Fifteen enzyme preparations examined under optimum equilibrium binding conditions gave estimated turnover numbers for the brine shrimp enzyme of 4050 ± 222 min^{-1} ($\bar{X}$ ± S.E.) for the Millipore method and 3210 ± 75 min^{-1} ($\bar{X}$ ± S.E.) for the centrifugation method. Since protein could be determined directly in the latter procedure, that turnover number is considered the more accurate estimate.

Binding of [^{14}C]ATP to the brine shrimp NaK-ATPase gave linear Scatchard plots, with an average dissociation constant for the complex of 0.12 ± 0.02 μM ($\bar{X}$ ± S.E., $n = 8$). The estimate turnover number from these studies was 3048 ± 233 min^{-1} ($\bar{X}$ ± S.E., $n = 8$).

Fig. 4 demonstrates the linearity of maximum binding for the ligands [γ-^{32}P]ATP and [^{14}C]ATP, as a function of the specific activity of each enzyme preparation. The regression line shown was fitted by the method of least squares to the phosphorylation data and had the equation $y = 0.31x + 0.02$ ($r^2 = 0.95$). The regression line for ATP binding (not shown) had the equation $y = 0.40x - 0.07$ ($r^2 = 0.92$). The turnover numbers from these studies are shown in Table III along with estimates of the binding capacity and apparent molecular weight of theoretically purified brine shrimp NaK-ATPase. It is apparent from these findings that the brine shrimp NaK-ATPase has an equivalent number of binding sites for phosphorylation and ATP.

Comparison of Kinetic Properties of Membrane and Partially Purified NaK-ATPase—The membrane and FT-membrane enzyme preparations produced similar temperature profiles with an optimum at 43°C. The enzyme activity was linear for 30 min at 42°C. Arrhenius plots of the temperature data revealed transitions in the enzyme activity which occurred at 27-28°C for both preparations. The membrane and FT-membrane preparations were also found to have similar pH profiles, monovalent cation activation curves, and K_m values for $MgCl_2$ and ATP. The optimum pH was 7.2. At a constant ionic concentration of 160 mM total Na plus K, the optimum Na:K ratio was 4:1, and at a constant Na:K ratio of 4:1, the enzyme was maximally active at 200 mM total Na plus K, with a second smaller peak in activity at 400 mM. The two peaks were reproducible and not due to a systematic error. The optimum concentration of ATP was found to be 5 mM, and

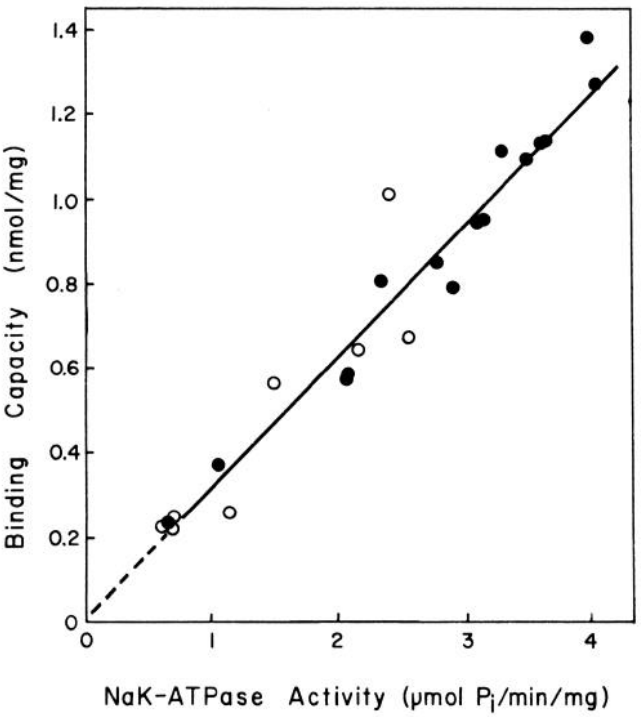

FIG. 4. Relationship between the measured binding capacity and the specific activity of different enzyme preparations from phosphorylation with [γ-^{32}P]ATP (●) and binding with [^{14}C]ATP (○). The *straight line* represents the regression line for the phosphorylation data fitted by the method of least squares ($y = 0.31x + 0.02$).

TABLE III

Ligand binding characteristics of the brine shrimp NaK-ATPase

Ligand	Turnover number[a]	Binding capacity[b]	Molecular weight[b]
	min^{-1}	*nmol/mg protein*	*g/mol*
[γ-^{32}P]ATP	3210 ± 75 (15)	4.32	232,000
[^{14}C]ATP	3048 ± 233 (8)	4.55	220,000

[a] Turnover numbers represented as $\bar{X}$ ± S.E. (n).

[b] The binding capacity and molecular weight are based on a theoretically pure enzyme with a specific activity of 833 μmol of P_i/h/mg of protein as projected from analyses of the SDS-polyacrylamide gels.

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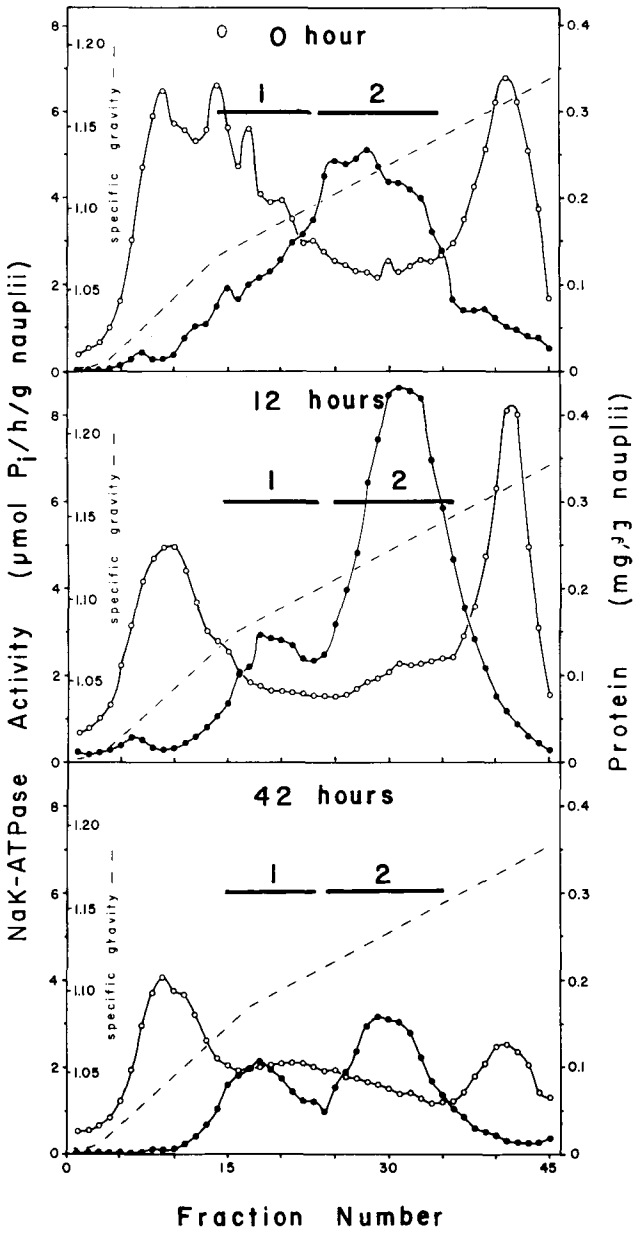

FIG. 5. Distribution of NaK-ATPase activity (●) and protein (○) in zonal sucrose density gradients of crude brine shrimp membranes prepared from nauplii at hatch (0 h) and at 12 and 42 h after hatch. Nauplii were synchronized to within 6 h of development by harvesting only those which hatched between 18 and 24 h incubation in sea water. The crude membranes were prepared from 70 to 75 g of nauplii (wet weight at time of synchronization) as described for the initial preparation of NaK-ATPase-rich membranes, except that the pellet from the first high speed centrifugation was used without further fractionation. The pellet contained about 1 g of protein and was resuspended in 90 ml of HM. The zonal experiments were done with a Beckman Ti-14 zonal rotor equipped with a B29 liner, and the sample and gradient were layered from the wall using a Technicon Auto Analyzer Model proportioning pump. The gradient consisted of 50 ml of distilled water overlay, 90 ml of sample, and 600 ml of a continuous 0.5 to 2.0 M sucrose gradient (approximately linear by volume). The gradient was prepared by pumping a reservoir of 275 ml of 2.0 M sucrose at 6.25 ml/min into a second mixing reservoir initially containing 325 ml of 0.5 M sucrose, which was pumped into the rotor at 12.5 ml/min. The gradient was centrifuged at 45,000 rpm for 90 min at 4°C in a Beckman L2-65B ultracentrifuge, after which 1-min fractions were collected at the rate of 12.5 ml/min. Light and heavy sedimenting NaK-ATPase membranes were collected by pooling the fractions shown by the *horizontal bars*.

the K_m determined from Lineweaver-Burk plots was 0.25 mM, when the Mg:ATP ratio was 2:1.

These kinetic characteristics for optimum enzyme activity are similar to those previously determined in more crude preparations from the nauplii of the brine shrimp (10), with the exception of the effect of total Na plus K. Enzyme assays had been run optimally at 400 mM total Na plus K at a constant Na:K ratio of 4:1, since two equivalent optima had been previously observed (at 250 and 400 mM) and the second arbitrarily chosen. The current findings indicate that the total Na plus K concentration should be near 200 mM for overall optimal activity.

Possible Isozymes of Brine Shrimp NaK-ATPase—The doublet banding pattern in the subunits on SDS-polyacrylamide gels and the two peaks in the monovalent ion stimulation open the intriguing question of whether isozymes of NaK-ATPase exist in the brine shrimp. Also, previous studies had shown that sucrose density gradient centrifugation separated

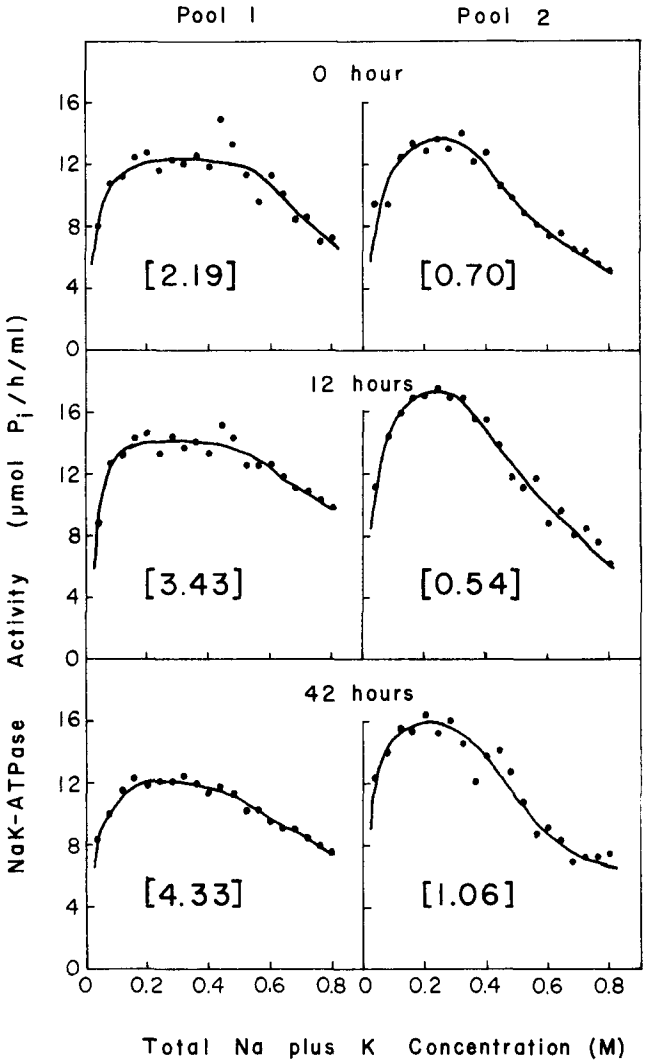

FIG. 6. Monovalent cation activation curves for the pooled NaK-ATPase membrane fractions isolated as described in Fig. 5. The ratio of Na to K was held constant at 4:1. The NaK-ATPase in each pool was further partially purified as described under "Materials and Methods," and the FT-membranes were subjected to SDS-polyacrylamide gel electrophoresis. The relative amount of each band in the doublet bands of the large subunit was determined by densitometry. The *bracketed numbers* within each curve represent the ratio of the slower to faster migrating bands of the large subunit doublet for each pool.

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two discrete zones of membranes containing NaK-ATPase. Crude membranes were isolated and fractionated in a zonal sucrose density gradient, to permit isolation and characterization of these two NaK-ATPase membranes. The sedimentation profiles of the NaK-ATPase membranes are shown in Fig. 5 for zonal experiments performed on crude membranes isolated from newly hatched nauplii and from nauplii 12 and 42 h after hatch. The sedimentation profile of the NaK-ATPase membranes from newly hatched nauplii was broad and ill defined, whereas after 12 h of further development, two discrete zones of NaK-ATPase membranes were evident. At 42 h after hatching, two NaK-ATPase zones clearly remained, but there was a loss in total enzyme activity which was most pronounced in the heavier sedimenting membranes. In the newly hatched nauplii, the protein was dispersed more throughout the gradient and a good portion of the NaK-ATPase membranes sedimented between the heavier and lighter zones of the older nauplii. For further characterization, the membranes were separated into two pools according to the fractions shown by the *horizontal bars* in Fig. 5. The monovalent cation activation curves and the ratio of the slower to faster bands of the large subunit doublet are shown in Fig. 6. With each developmental stage, the lighter membranes showed a broad flat total monovalent cation activation profile for the NaK-ATPase and a dominance in the slower protein band of the large subunit doublet, while the heavier membranes showed a narrow monovalent cation activation profile and a dominance of the faster band of the large subunit doublet. In *Pool 1* (lighter membranes), the NaK-ATPase activity was equivalent at 200 and 400 mM Na plus K, whereas in *Pool 2* (heavier membranes), the relative activity at 400 mM Na plus K was much reduced.

DISCUSSION

Partial Purification of Brine Shrimp NaK-ATPase—Partial purification of the naupliar brine shrimp NaK-ATPase was achieved by deoxycholate treatment of isolated membranes followed by a freeze-thaw procedure. The deoxycholate treatment produced a 1.5-fold activation of the enzyme (after removal of free deoxycholate) and a 2- to 3-fold purification over that of the membrane enzyme preparation. This is similar to the deoxycholate purification reported by Lane *et al.* (5) for the dog kidney NaK-ATPase and by Pitts *et al.* (23) for the dog heart NaK-ATPase, but without the accompanying "solubilization." The freeze-thaw procedure produced an additional 2- to 3-fold purification. Freezing at −12°C apparently caused a rearrangement of the membranes, resulting in the appearance of a population of NaK-ATPase enriched light vesicles (Fig. 1*A*) which could be recovered from heavier membrane aggregate arrays of lower specific activity (Fig. 1*B*). The complex membrane aggregate arrays were not apparent in crude membranes (10). The enzyme-rich vesicles are presumably formed as a result of protein aggregation during slow freezing (24–26).

The overall purification of the naupliar brine shrimp NaK-ATPase is 100- to 200-fold over the homogenate. This is as high or higher than any purification reported for other tissue sources of the NaK-ATPase (4–9, 27, 28). The yield of the partially purified brine shrimp NaK-ATPase is about 10 mg/200 g, wet weight, of nauplii. The partially purified beef brain (27) and beef heart (28) preparations have yields of 6 and 15 mg/200 g of starting material, respectively. The purified preparations usually show yields of 20 to 30 mg for a comparable amount of starting material (5–8), although yields of 1 mg and 5 mg are reported by Nakao *et al.* (29) and Kyte (4), respectively. The brine shrimp preparation is stable and apparently exists in a vesicular form (Fig. 1).

Molecular and Kinetic Properties of Brine Shrimp NaK-ATPase—Two major proteins are observed to enrich during purification (Fig. 2). One is a 100,000-dalton protein identified as the large catalytic subunit by its potassium-sensitive, (Na + Mg)-ATP-dependent phosphorylation capacity (Fig. 3); the other is a 40,000-dalton protein identified as the small glycoprotein subunit (Fig. 2*E*). These properties are in agreement with similar findings for other NaK-ATPase preparations (See Table IV), and confirm that the specific protein complex of the brine shrimp NaK-ATPase can be identified in our preparations. Moreover, the kinetic characteristics of the membrane and partially purified FT-membrane enzyme are identical, demonstrating that the NaK-ATPase has not been altered during purification.

The molecular properties of the naupliar brine shrimp NaK-ATPase are summarized and compared in Table IV with those from other sources of the enzyme. The molecular weight of the large subunit from the brine shrimp is within the range of values observed in other species. The small subunit (glycoprotein) of the brine shrimp, however, is notably smaller than all other preparations. The molecular weight of some glycoproteins have been reported to be overestimated by SDS-polyacrylamide gel electrophoresis (30). Kyte (31) reported molecular weights of 127,000 and 45,000 for the large and small subunits of the dog kidney NaK-ATPase when analyzed by SDS gel filtration. However, Hopkins *et al.* (9) found essentially the same molecular weights for the two subunits of the duck salt gland NaK-ATPase using either SDS gel filtration or SDS-polyacrylamide gel electrophoresis. Thus, anomalous behavior in SDS-polyacrylamide gels does not appear sufficient to account for the species differences.

The mass ratio of the large to small NaK-ATPase subunits in the brine shrimp preparation is 2:1, corresponding to an apparent molar ratio of 1:1. These figures are in agreement with those from the dog kidney (5, 33) and beef heart (28), whereas with other preparations the molar ratio is close to 2:1 (Table IV). Recently, Marshall (37) measured the mass ratio in the dogfish rectal gland NaK-ATPase purified by deoxycholate rather than the Lubrol method of Hokin's group (7) and found a ratio of 1.58, one-half that of Hokin's findings. The two preparations have similar specific activities and both were analyzed under conditions within which the gel scans obeyed Beer's law. However, Marshall does not provide evidence to assess the purity of the isolated subunits in his preparation, which is a critical consideration (19). In the brine shrimp NaK-ATPase, there is no significant change in the mass ratio for preparations between 200 and 475 μmol of P_i/h/mg of protein, suggesting that the two subunits are largely free of contamination when separated on SDS gels. Prolonged electrophoresis (Fig. 2*D*) which clearly resolves the double bands of the large subunit (see below) shows no additional contaminating protein bands in this region. The species discrepancies may be due to differences in the extinction for Coomassie blue by different proteins, especially glycoproteins (17, 38, 39) or inaccurate molecular weight determinations (or both).

Estimated from the ratio of activity to ligand binding, the turnover number for the brine shrimp NaK-ATPase ranges between 3050 and 3200 min^{-1} depending on the ligand (Table III). The turnover number as well as the specific activity of the theoretically pure brine shrimp NaK-ATPase (833 μmol of P_i/h/mg of protein) are lower than that of most other preparations (Table IV). However, if there are two NaK-ATPase isozymes in the brine shrimp with different kinetic properties (see below), only one may be optimally measured

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TABLE IV

Comparison of molecular properties of NaK-ATPase preparations from different sources

Source	Subunit M_r[a]		Subunit ratio[b] (large/small)		Estimated[c] specific activity at purity	Ligand binding data[d]						Reference
						[γ-^{32}P]ATP		[^{3}H]Ouabain		[^{14}C]ATP		
	Large	Small	Mass	Molar		M.A.	M_r	M.A.	M_r	M.A.	M_r	
	g/mol				*μmol P_i/h/mg protein*	*min^{-1}*	*$g \cdot 10^{-3}$/mol*	*min^{-1}*	*$g \cdot 10^{-3}$/mol*	*min^{-1}*	*$g \cdot 10^{-3}$/mol*	
Rabbit kidney	96,000	57,000	2.8	1.66	2,200		280	8,800	240	9,100	250	6, 32
Dog kidney	84,000	57,000	1.62	1.10	890	3,000	202	2,900[e]	196[e]			4, 33, 34
Dog kidney	89,000	56,000	1.88	1.18	1,550			6,480	250			5
Pig brain	100,000				6,000	12,200	120					35
Beef heart	94,000	58,000	2.08	1.28	1,150	8,920	496	8,420	468			28
Duck salt gland	94,000	60,000	2.83	1.82	2,300	5,460	142	10,000	278			9
Eel electric organ	93,000	47,000	3.57	1.85	2,000	7,270	218	7,370	221			8, 36
Dogfish rectal gland	97,000	55,000	3.82	2.17	1,580	5,900	224	6,140	233			7
Brine Shrimp	95,000–101,000	38,000–40,000	1.99	0.80	833	3,210	232			3,048	220	This paper

[a] Subunit molecular weights (M_r) are those obtained by SDS-polyacrylamide gel electrophoresis.

[b] The mass ratios of the large to small subunits are based on quantitative scanning of the SDS gels. The molar ratios are calculated from the above shown molecular weights and mass ratios.

[c] The estimated specific activity of a theoretically purified enzyme preparation is calculated from the relative purity estimated from SDS gels and the maximum specific activity reported for each preparation.

[d] The molecular activity or turnover number (M.A.) and the estimated specific activity of a theoretically purified enzyme are used to calculate the molecular weight (M_r) estimate for the holoenzyme.

[e] Not from [^{3}H]ouabain binding, but from kinetic titration with *N*-(4′-amino-*n*-butyl)-3-aminoacetylstrophanthidin.

and the specific activity and molecular activity would be low. It is also difficult to compare specific and molecular activities between different preparations as these are sensitive to assay methods and any inactivation incurred during purification (*cf.* dog kidney preparations in Table IV). Further complications associated with the determination of the molecular activity of NaK-ATPase have been reviewed by Nørby and Jensen (40).

On the other hand, estimates of the molecular weight of the brine shrimp NaK-ATPase from the binding data are in close agreement with those of other preparations (range, 200,000 to 280,000, Table IV). Radiation inactivation studies indicate an average molecular weight of 250,000 (range, 190,000 to 300,000) as determined from several species including a crustacean (41). A value of 280,000 has been obtained by gel filtration of the detergent solubilized crustacean nerve NaK-ATPase (42) and pig brain NaK-ATPase (43). Higher estimates have also been obtained by these methods, but probably do not represent the minimum molecular weight (2, 41). Molecular weights determined from binding data measure the protein molecular weight, whereas radiation inactivation and particularly gel filtration would be sensitive to the presence of lipids and thus should give slightly higher estimates, as lipids are required for activity (1, 2).

The binding data for the brine shrimp NaK-ATPase indicate that there is one phosphorylation site per ATP binding site. This agrees with most of the preparations shown in Table IV, as well as others (44–47). In the duck salt gland NaK-ATPase, however, there are two apparent phosphorylation sites per ouabain binding site (9). Albers *et al.* (44) also reported two phosphorylation sites per ouabain binding site in the electric eel, although this does not agree with the more recent findings of Perrone *et al.* (36) using the purified eel enzyme. Current evidence indicates that the phosphorylation site (27), the ouabain binding site (48, 49), and presumably the nucleotide binding site are all on the large subunit and that the NaK-ATPase holoenzyme contains at least two large subunits (50, 51). Thus, each enzyme unit should have two of each type of binding site. Recent evidence from trypsin digestion studies suggest that ATP may bind to one of the large subunits and phosphorylate the other (32). The present data are consistent with "half-of-the-sites reactivity" mechanism proposed for the NaK-ATPase (52, 53).

An interesting feature of the brine shrimp NaK-ATPase preparation is that the subunits, unlike those from other tissues, are double-banded (Figs. 2 and 3). The doublet banding of the large and small subunits is observed in gels prepared with different buffers, pH, denaturants, and acrylamide concentrations, whereas other proteins in the same gel are not double-banded. Thus, the double banding is a property of the enzyme preparation and not an artifact of the gel system. Furthermore, it is unlikely that the doublet banding is due to oxidation or proteolysis during preparation as these processes usually result in immediate inactivation (32, 51). The brine shrimp enzyme is stable at 0°C for 1 week and is prepared and maintained in the presence of 2-mercaptoethanol, and both bands of the large subunit are phosphorylated from ATP (Fig. 3). There is a positive and significant ($p < 0.001$) correlation in the relative amounts of the slower and faster migrating bands between the large and small subunits. Thus, in a given holoenzyme, the slower band of the large subunit may be expected to be found with the slower band of the small subunit (and likewise for the faster bands).

In an earlier study (10), we reported that the brine shrimp NaK-ATPase containing membranes could be separated into two peaks by sucrose density gradient centrifugation and that at a constant Na:K ratio of 4:1, there were two kinetic optima for total Na plus K. These observations were confirmed in this study (Figs. 5 and 6). Moreover, the heavier sedimenting NaK-ATPase membranes were found to display predominantly the lower total ionic strength optimum, while the lighter sedimenting NaK-ATPase membranes had a broad flat ionic strength profile, similar to that observed previously (10). In the previous study, we used younger animals (36 h) which apparently contained a proportionately greater amount of lighter membranes. The lighter sedimenting NaK-ATPase membranes after partial purification were found to be enriched in the slower migrating band of the large subunit doublet, and vice versa for the heavier membranes. These correlations strongly suggest the presence of at least two NaK-ATPase isozymes in the brine shrimp nauplius. Furthermore,

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the lighter NaK-ATPase membranes may represent a developmentally early form of the NaK-ATPase. If this is the case, the pattern of the doublets in the lighter and heavier NaK-ATPase membranes suggest that the isozymes may be precursor-products, which would have an important bearing on the study of the biogenesis of the brine shrimp NaK-ATPase. This aspect is under continued investigation.

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